ISSN: 2755-0281

Journal of Pediatrics Research Reviews & Reports

SCIENTIFIC Research and Community

Research Article

Open Access

Interleukin-10 Gene Expression Predicts Response to Therapy in Childhood Acute Lymphoblastic Leukemia

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ABSTRACT

Several studies have demonstrated that IL-10 have been associated with therapy outcome in hematological malignancies. IL-10 might therefore also correlate with clinical outcome in childhood acute lymphoblastic leukemia (ALL). In addition to expressing full-length IL-10, a new splicing-derived IL-10 variant (termed IL-10δ3) can be detected in ALL samples. These isoforms generally have different affinities to their receptors, contributing to generating important function in modulating the biologic activity of normal IL-10.

Most childhood malignancies lack reliable and specific biochemical tumor markers correlating with disease extent and aggressiveness. That is why several non-specific indirect indicators, including ESR, CRP and LDH, are used in common practice as additional diagnostic and prognostic tools. The study presented here aimed to assess whether the expression of IL-10 and its isoform, IL-10δ3 correlate with other prognostic factors. Mainly, the response to treatment and risk of relapse in 60 children with B-cell precursor (BCP)-ALL, treated at the Pediatric Oncology Department, National Cancer Institute, Cairo University and Damnhour Teaching hospital (DMNI) during the time period from August 2009 to May 2014 according to NCI protocols modified from the St. Jude Total XV protocols. The median age of the patients was 6 years (range, 0.91 years to 24 years) and the male: female ratio was 1.61:1 (37 males and 23 females).

Study data showed a possible role of IL-10δ3 particularly decreased incidence of relapse and prolonged event-free survival in first complete remission, whereas no association between response to therapy and IL-10 and IL-10 isoform, IL-10δ3, expression. Moreover, CD34 expression with a good prognosis was higher in patients displaying IL-10δ3 expression compared to patients not expressing the isoform.

Results revealed correlation between pro-B stage and IL-10δ3 expression (the 4 patients with pro-B stage expressed IL-10δ3). This relation was not clear since the pro-B immunophenotype is often associated with unfavorable outcome and IL-10δ3 was linked to better clinical outcomes and prolonged event-free survival at relapse and first complete remission. May be IL-10δ3 expression reversed bad prognosis.

In childhood B-cell precursor (BCP)-ALL, IL-10 isoform, IL-10δ3, expression was associated with a favorable prognosis, better event-free survival and decreased the incidence of relapse in first complete remission.

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Received: November 30, 2025; **Accepted:** December 08, 2025; **Published:** January 19, 2026

Introduction

The link between inflammation and the development of cancer has been recognized since 1863, when Rudolf Virchow discovered leukocytes in neoplastic tissues and made the first relation between inflammation and cancer [1]. Since then, a number of cancers have been linked to inflammatory origins, and in many cases it has been considered how the tumor microenviron-ment highly resembles an inflammatory site [2-4].

Interleukin-10 (IL-10 or IL10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. In humans IL-10 is encoded by the IL10 gene [5]. A main function of IL10 is to suppress NFκB, which leads to reduced levels of the pro-inflammatory cytokines TNFα, IL6 and IL12 [6]. Furthermore, IL-10 down-regulates the expression of major histocompatibility complex (MHC) class II antigens in monocytes and dendritic cells, while it enhances it on B cells [7,8].

The role of IL-10 in tumor development remains unclear, and its production in the tumor microenvironment is controversial. In spite of this, elevated IL-10 levels in peripheral blood samples have been related to a worse prognosis but high levels in tumor

samples to a better prognosis [9]. Moreover, IL-10 polymorphisms by single nucleotide polymorphism (SNP) were analyzed and shown to be in association with the susceptibility to cancers; some solid tumors (e.g, malignant melanoma, prostate cancer, and breast cancer) and hematologic malignancies (e.g, multiple myeloma, non-Hodgkin's lymphoma, AML, and ALL) [10-13].

Aim of the Work

The general aims of the study were to evaluate new possible prognostic factors in childhood ALL, and, in the context of contemporary intensive treatment, to assess the value of established prognostic factors.

The Specific Aims in this Study were as Follows

- To detect interleukin-10 expression [full-length IL-10 and a new splicing-derived IL-10 variant termed IL-10δ3] and its value in predicting the early response to treatment in childhood B-cell precursor (BCP)- ALL.
- To study correlation between interleukin-10 expression and other prognostic factors as age, sex, TLC, and hemoglobin level in childhood B-cell precursor (BCP)-ALL.

Patients and Methods

This is a retrospective analysis of 60 consecutive pediatric patients with a documented diagnosis of B-cell precursor (BCP)-ALL, treated at the Pediatric Oncology Department, National Cancer Institute, Cairo University and Damnhour Teaching hospital (DMNI) during the time period from August 2009 to May 2014. The median age of the patients was 6 years (range, 0.91 years to 24 years) and the male: female ratio was 1.61:1 (37 males and 23 females). All patients were diagnosed according to standard methods including blood picture, bone marrow, cytochemistry and immunophenotyping. Median follow-up time was 37.96 months (range, 1 to 70 months).

Treatment Methods

Patients were treated according to NCI childhood ALL protocols modified from the St. Jude Total XV protocols (some patients were treated according to St. Jude Total XIII protocol). Infants (≤12 months) were treated according to infantile protocol (interfant 99).

Definitions

Complete remission (CR) was defined as the absence of leukemic blasts in peripheral blood and CSF and less than 5% blasts in BM, together with hematopoietic regeneration and no evidence of extramedullary (localized) disease. Relapse was defined as recurrence of more than 5% lymphoblasts or localized leukemic infiltrates at any site. The duration of event free survival (EFS) was calculated from the first day of treatment until the date of first event [death, relapse, second malignancy and induction failures such as failure to achieve complete remission by day 42 (non-response), or death during induction therapy], or the date of last follow-up. Induction death and non-response were considered failures at time zero. The observation time was censored at the last follow-up date if no events occurred.

Risk Classification

Patients were classified into, low risk (LR), standard risk (SR) and high risk (HR) groups. The main criteria for stratification of B-cell precursor (BCP)-ALL patients into particular risk groups were based on presenting characteristics of the patients and treatment response (Table 1). Patients LR and SR usually have favorable outcome while the HR usually have unfavorable outcome.

Table 1: Risk Classification System in St. Jude Total Therapy Study

Risk Group	Features
Low	B-ALL with age 1-9 years and presenting leukocyte count of less than 50×109/L; translocation t (12;21) (ETV6-RUNX1); or hyperdiploidy >50 (DNA index of 1.16 or more; of 1.60 or less). Must not have (I) CNS leukemia (CNS-3 status); (II) testicular leukemia; (III) t (9;22), t (1;19); (IV) rearranged MLL gene; (V) hypodiploidy; or (VI) poor early response*.
Standard	B-cell precursor cases not classified as low or high risk.
High	(1) Patients with t (9;22) (BCR-ABL1) or poor early response; or (2) induction failure.

* (>5% Blasts in Bone Marrow on Day 15 and/or ≥ 0.01% Blasts on Day 42).

All patients were subjected to a full clinical, radiological and laboratory investigation including chest X Ray, abdominal Ultrasound, CSF examination and a full chemistry profile including liver and kidney function tests, complete blood count (CBC) and Bone Marrow aspirate. Immunophenotyping was done using monoclonal antibodies which were analyzed on Coulter XL (Panel included CD1, CD2, CD3, CD4,CD5, CD7, CD8, CD10, CD19, CD22, Cytoplasmic μ, anti κ, anti λ, CD13, CD33, CD34, anti-class II MHC and TDT).

Sampling

Peripheral blood/Bone marrow EDTA samples were obtained for RNA preparation.

RNA Extraction, Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA Extraction was done using QIAamp RNA Blood Mini Kit (Catalog no. 52304). The quantification and purity of RNA were determined by measuring the absorbance at 260nm (A260) using a nanodrop spectrophotometer (ND - 1000). Pure RNA with an A260 / A280 ratio of 1.9 – 2.1 was used. The integrity and size distribution of total RNA purified was checked by running on 1% agarose gel electrophoresis and ethidium bromide staining. The GeneAmpGold RNA PCR Reagent Kit (P/N 4308206 Applied Biosystems) was used for the reverse transcription (RT) and polymerase chain reaction (PCR) amplification of the specific target gene from total RNA. First-strand cDNA was synthesized from 2 μg of RNA using RT Reaction. The integrity of cDNA for all cases was tested by amplifying the house keeping gene, β actin.

For the detection of IL-10 splice variants, the sequence of IL-10 primer pair used was, sense primer: ATGCACAGCTCAGCACTGCT; IL-10 antisense primer: TCAGTTTCGTA-TCTTCATTGTCAT. A 50 μL PCR reaction mix was prepared containing 9.4 μL 5X RT-PCR

Buffer, 3.2 µL 25 mM Magnesium Chloride, 3.7 µL 10 mM dNTP Blend [200 µM of each dNTP; (dATP, dCTP, dGTP, and dTTP)], 0.5 µL AmpliTaq Gold DNA Polymerase (2.5 Units), 0.5 µL IL-10 sense primer (100 pmol/ µL), 0.5 µL IL-10 antisense primer (100 pmol/ µL), 100 ng cDNA and to 50 µL Nuclease-free water. Amplification was done in the Thermal Cycler, cycle conditions were as follows: initial denaturation at 94°C for 10 minutes, followed by 35 cycles at 94°C for 45 seconds, 62°C for 30 seconds, 72°C for 1.25 minutes, and a final elongation at 72°C for 10 minutes. The amplification products were separated by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. IL-10 was detected at 537 bp, while isoform IL-10δ3 at 384 bp (Figure.1 A& B). All assays were done in duplicate. BM samples negative for IL-10 δ3 expression were confirmed by nested PCR.

Statistical Analysis

Frequencies were calculated for descriptive purposes. Differences in the distribution of categorical variables were analyzed by $\chi 2$ or Fisher's exact test if the frequencies were small (<5) and by Mann–Whitney U for non-paired data test. To verify the hypothesis of differences in mean values among the independent groups the ANOVA analysis of variance was used. Analysis of the probabilities of event-free survival (pEFS) was performed using the Kaplan–Meier method and the groups were compared with the log-rank test. Statistical analysis were done by the SPSS Software for Windows (version 18; portable SPSS). Significance was set to $P < 0.05$ and highly significant to $P < 0.01$.

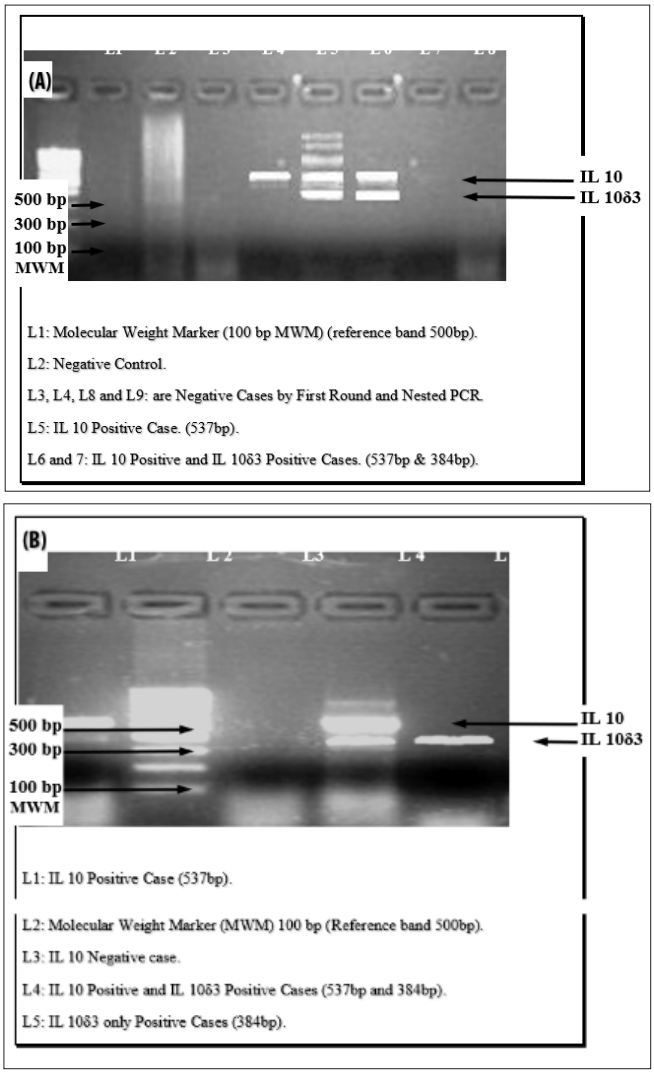

Figure 1: Expression of IL-10 and Isoform IL-10δ3 in BCP-ALL

Results

The study comprised 60 pediatric patients with B-cell precursor (BCP)-ALL. two patients were excluded from the study because their parents decided to seek help elsewhere and did not return to Pediatric Oncology Department, NCI [one case was a 17 years old female not expressing IL-10, the other was a 11 months old male who exclusively expressed the IL-10.

Patients Presenting to the NCI, Eligible Numbers and Numbers Excluded

The median age of the patients was 6 years (range, 0.91 years to 24 years) and the male: female ratio was 1.61:1 (37 males and 23 females). IL-10 and IL-10 isoform, IL-10δ3, expression was measured for all patients to assess its possible prognostic significance on treatment outcome.

Within the total number of 60 patients with a documented diagnosis of precursor B-ALL, Full-length IL-10 transcript was expressed in 51.6% (31/60) of cases and was the sole variant in 20% (12/60) of cases. The frequency of IL-10δ3 expression was 38.3% (23/60) of cases. Only four cases (6.6%) expressed IL-10δ3 exclusively (Table 2). In 41.6% (25/60) of cases, IL-10 expression was below detection limit.

Table 2: Features of the 4 (BCP)-ALL Patients who Expressed IL-10δ3 Exclusively

	IL-10δ3			
	Case 1	**Case 2**	**Case 3**	**Case 4**
Risk Classification	SR	SR	SR	SR
Age	10	8	11	6
Sex	F	M	M	F
TLC (×109/L)	6.77	101	67	57.47
Liver Edge Below Costal Margin (cm)	2	Free	2	Free
Spleen Edge Below Costal Margin (cm)	3	Free	6	Free
Immunophenotype	Pro B	Pre B	Pro B	CALL
CD19 Positivity	Yes	Yes	Yes	Yes
CD22 Positivity	No	Yes	Yes	No
CD10 Positivity	No	Yes	No	Yes
CD34 Positivity	Yes	Yes	Yes	Yes
MHC CLASS II	No	Yes	Yes	No
DNA Index	Nd	1	1	1.16
Serum LDH (U/L)	Nd	788	Nd	1184
Remission Achieved	Yes	Yes	Yes	Yes
Relapse	No	No	No	No
Death	No	No	No	No
EFS (months)	27	44.13	43.66	70
OS (months)	27	44.13	43.66	70
Others	11 q23 Rearran-Gement	CNS III	CNS IA	CNS IA

Abbreviations:
ND: Not Determined
SR: Standard Risk
EFS: Event-free Survival
OS: Overall Survival

Table 3: Characteristics of Pediatric B-cell Precursor (BCP)-ALL Cases Studied for IL-10 Expression

	IL-10 Expression			**Total**	**P Value 1**
	None (n=24)	**IL-10 (n=11)**	**IL-10δ3 (n=23)**		
RISK Classification					
LR	5(20.8%)	4(36.4%)	7(30.4%)	16(27.6%)	
SR	15(62.5%)	7(63.6%)	12(52.2%)	34(58.6%)	
HR	4(16.7%)	—	3(13%)	7(12.1%)	0.632
Age(years)					
< 1	—	—	1(4.3%)	1(1.7%)	
1-10	17(70.8%)	7(63.6%)	17(73.9%)	41(70.7%)	
> 10	7(29.2%)	4(36.4%)	5(21.7%)	16(27.6%)	0.690
Mean ± S.D Median (range)	8.81 ± 5.97 9(2-24)	8.05 ± 4.40 6(2.58-16)	7.12 ± 4.72 6(0.91-17)		0.544
Sex					
Male	17(70.8%)	5(45.5%)	14(60.9%)	36(62.1%)	
female	7(29.2%)	6(54.5%)	9(39.1%)	22(37.9%)	0.352
Total Leukocyte Count (×109/L)					

< 50	19(79.2%)	7(63.6%)	16(69.6%)	42(72.4%)	
50-100	2(8.3%)	2(18.2%)	3(13%)	7(12.1%)	
> 100	3(12.5%)	2(18.2%)	4(17.4)	9(15.5%)	0.882
Mean ± S.D Median (range)	38.34 ± 70.18 8.72(1.51-308)	56.19 ± 74.39 30(5.6-260)	44.78 ± 72.65 13.4(1.8-325.2)		0.793
Liver Edge Below Costal Margin (cm)					
Free	14(58.3%)	6(54.5%)	12(52.2%)	32(55.2%)	
1-4	8(33.3%)	5(45.5%)	6(26.1%)	19(32.7%)	0.364
≥5	2(8.3%)	—	5(21.7%)	7(12.1%)	
Spleen Edge Below Costal Margin (cm)					
Free	11(45.8%)	6(54.5%)	12(52.2%)	29(50%)	
1-4	9(37.5%)	3(27.3%)	7(30.4%)	19(32.8%)	
≥5	4(16.7%)	2(18.2%)	4(17.4%)	10(17.2%)	0.977
Immunophenotype					
Pro B	—	—	4(17.4%)	4(6.9%)	
CALL	13(54.2%)	6(54.5%)	9(39.1%)	28(48.3%)	0.149
Pre B	11(45.8%)	5(45.5%)	10(43.5%)	26(44.8%)	
DNA index					
< 1.16	12(100%)	1(50%)	12(80%)	25(86.2%)	
≥ 1.16	—	1(50%)	3(20%)	4(13.8%)	0.099
Serum LDH (U/L)					
Mean ± S.D Median (range)	2207.67 ± 2152.15 971(367-7031)	1779.50 ± 2900.11 677(307-7683)	2444.25 ± 3513.48 874.5(525-12000)		0.897
CD19 Positivity					
No	5(20.8%)	4(36.4%)	2(8.7%)	11(19%)	
Yes	19(79.2%)	7(64.6%)	21(91.3%)	47(81%)	0.150
CD10 Positivity					
No	6(25%)	5(45.5%)	6(26.1%)	17(29.3%)	
Yes	18(75%)	6(54.5%)	17(73.9%)	41(70.7%)	0.424
CD22 Positivity					
No	6(25%)	4(36.4%)	5(21.7%)	15(25.9%)	
Yes	18(75%)	7(64.6%)	18(78.3%)	43(74.1%)	0.655
CD34 Positivity					
No	19(79.2%)	9(81.8%)	12(52.2%)	40(69%)	
Yes	5(20.8%)	2(18.2%)	11(47.8%)	18(31%)	0.080
MHC CLASS II					
No	9(37.5%)	5(45.5%)	7(30.4%)	21(36.2%)	
Yes	15(62.5%)	6(54.5%)	16(69.6%)	37(63.8%)	0.685
Response to Therapy					
Responders	21(87.5%)	11(100%)	23(100%)	55(94.8%)	
Non-Responders	2(8.3%)	—	—	2(3.5%)	0.345
Induction Death	1(4.2%)	—	—	1(1.7%)	
Relapse					
None	18(75%)	8(72.7%)	21(91.3%)	47(81%)	
Relapse	6(25%)	3(27.3%)	2(8.7%)	11(19%)	0.267
EFS ‡ (months)					
Mean ± S.D					
Mean ± S.D Median (range)	17.66 ± 16.53 8(0-42)	25.58 ± 15.57 27(5.4-50.5)	34.57 ± 17.54 41(1-70)		0.005

Abbreviations:
EFS: Event-Free Survival
IL-10: Interleukin-10
IL-10δ3: Splicing-Derived Il-10 variant
LDH: Lactate Dehydrogenase
LR: Low Risk
SR: Standard Risk,
HR: High Risk
S.D: Standard Deviation

Significant differences were assessed by $\chi 2$.
‡ Significant differences between IL-10 expression between groups were assessed by ANOVA analysis.

Of the 60 (37 Males and 23 Females) Cases of Precursor B-ALL, 58 Patients (36 Males and 22 Females) were Eligible for Statistical Analysis. Their Characteristics were Recorded in Table 3.

- Liver was enlarged in 10/24 patients (41.7%) not expressing the IL-10, 5/11 patients (45.5%) who exclusively expressed the IL-10 and 11/23 patients (47.8%) having the IL-10δ3 variant. Hepatomegaly among different groups was not statistically significant.
- Spleen was enlarged in 13/24 patients (54.2%) not expressing the IL-10, 5/11 patients (45.5%) who exclusively expressed the IL-10 and 11/23 patients (47.8%) expressing the IL-10δ3 variant. Splenomegaly among different groups was not statistically significant.

Risk Classification

- In patients with IL-10δ3 variant, 1/23 patient (4.3%) was classified as high/infant (≤12 months) and treated according to infantile protocol (interfant 99).
- As for the LRG, it included 5/24 (20.8%) patients not expressing the IL-10, 4/11 patients (36.4%) exclusively expressed the IL-10 and 7/23 patients (30.4%) expressed the IL-10δ3 variant.
- In the SRG there were, 15/24 patients (62.5%) not expressing the IL-10, 7/11 patients (63.5%) exclusively expressed the IL-10 and 12/23 (52.2%) that expressed the IL-10δ3 variant.
- The HRG had 4/24 patients (16.7%) not expressing the IL-10 and 3/23 patients (13%) expressed the IL-10δ3 variant while there were no patients (0%) who exclusively expressed the IL-10 in this group. No statistically significant difference was observed among the 3 risk groups and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Outcome and Relapse Rate

Within the total number of 58 evaluable patients included in this study, six patients died (10.3%). One patient not expressing the IL-10 died of treatment-related death, another patient exclusively expressed the IL-10 died of disease progression, and four patients died of relapsed ALL [three patients not expressing the IL-10 and one patient exclusively expressed the IL-10.

Relapse occurred in 18.9% (11/58) patients after achieving complete remission, 6/24 patients (25%) not expressing the IL-10, 3/11 patients (27.3%) exclusively expressed the IL-10 and 2/23 patients (8.7%) expressed the IL-10δ3 variant (Figure. 2)

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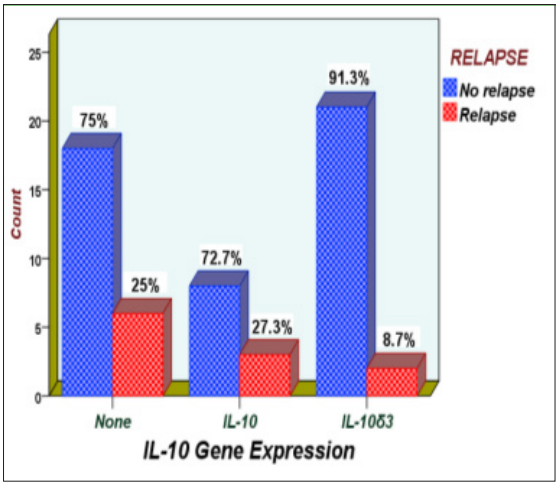

Figure 2: Incidence of Relapse Based on Expression of Il-10 and Il-10 Isoform, Il-10δ3, in Pediatric B-Cell Precursor (BCP) Acute Lymphoblastic Leukemia

Event Free Survival (EFS)

The median value of event-free survival (EFS) was 8 months in patients not expressing the IL-10, 27 months in patients exclusively expressed the IL-10 and 41 months in patients who expressed the IL-10δ3 variant (Figure. 3). The median value of EFS was statistically highly significant (P= 0.005).

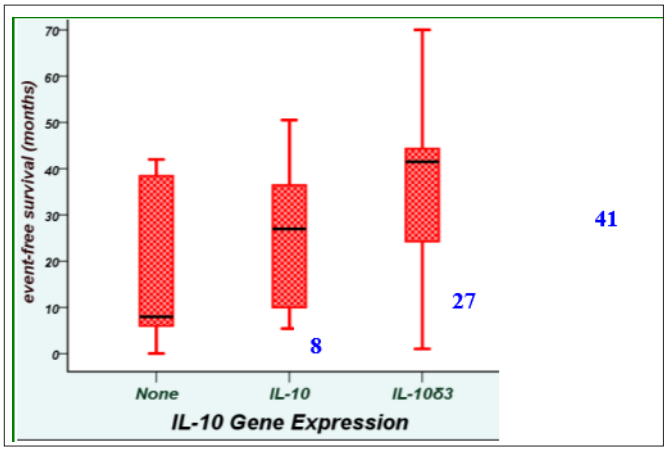

Figure 3: Gene Expression of Il-10 and Event-Free Survival (EFS) in Pediatric B-Cell Precursor (BCP) Acute Lymphoblastic Leukemia. The Cross-Line Denotes the Median, and the Lower and Upper Whiskers Represent the Downmost Value and the Upmost Value, Respectively

In the analysis of probabilities of event-free survival (pEFS) (Figure. 4), patients with IL-10δ3 expression had a significantly better pEFS at five years compared with patients expressing only the normal IL-10 transcript and/or patients expressing IL-10 below detection limit (P= 0.047).

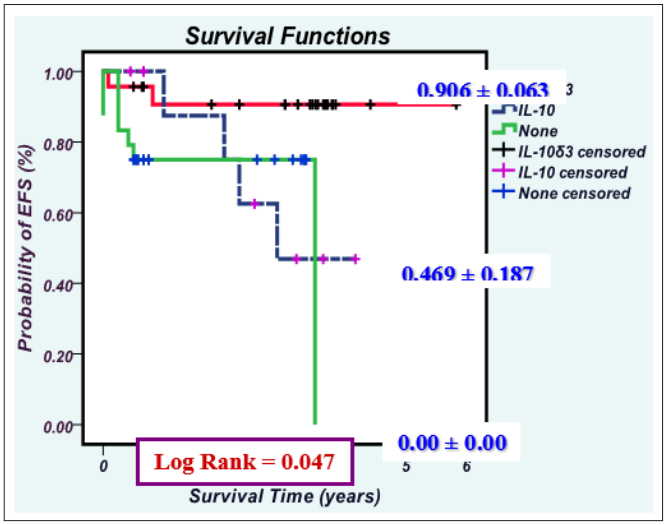

Figure 4: Correlations Between Il-10 Expression and Probabilities of Event-Free Survival (PEFS) in Pediatric B-Cell Precursor (BCP) Acute Lymphoblastic Leukemia

- (pEFS) depending on the expression of IL10δ3 compared with patients expressing only the normal IL-10 transcript was statistically significant (Log Rank = 0.034).
- (pEFS) depending on the expression of IL10δ3 compared with patients not expressed the IL-10 (None) was statistically significant (Log Rank = 0.026).
- (pEFS) depending on patients expressing only the normal IL-10 transcript compared with patients not expressed the IL-10 (None) was not statistically significant (Log Rank = 0.83).

Correlation of IL-10 Expression with Different Prognostic Factors

Age

Patients were divided into 3 groups, younger than 1 year, those older than 10 years who usually have a poor prognosis, and then patients between 1 to 10 years who usually have better prognosis. No statistically significant difference was observed among the 3 groups and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

In patients not expressing the IL-10, the age ranged from 2 to 24 years with a mean of 8.81 and median 9 years, while the age ranged from 2.58 to 16 years with a mean of 8.05 and median 6 years in patients exclusively expressed the IL-10. The age range was from 0.91 to 17 years with a mean of 7.12 and median 6 years in patients who expressed the IL-10δ3 variant. There was no statistically significant relation between the age and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Sex

In females, 7/24 (29.2%) patients did not express the IL-10, 6/11 (54.5%) patients exclusively expressed the IL-10 and 9/23 (39.1%) patients expressed the IL-10δ3 variant while in males, 17/24 (80.8%) patients did not expressed the IL-10, 5/11 (45.5%) patients exclusively expressed the IL-10 and 14/23 (60.9%) patients expressed the IL-10δ3 variant. There was no statistically significant relation between the sex and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Total Leucocytic Count (TLC)

Patients were divided into 3 groups, a group with TLC <50 ($\times 10^9$/L) who usually have a better prognosis, a group with TLC from 50 to 100 ($\times 10^9$/L) and a group with TLC >100 ($\times 10^9$/L) who usually have poor prognosis. No statistically significant difference was observed among the 3 groups and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

The mean value of TLC was 38.34 ($\times 10^9$/L) in patients not expressing the IL-10, 56.19 ($\times 10^9$/L) in patients exclusively expressed the IL-10 and 44.78 ($\times 10^9$/L) in patients who expressed the IL-10δ3 variant. There was no statistically significant relation between TLC and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Immunophenotyping

- Within the total number of 58 patients included in this study, they were 4/58 (6.9%) pro-B patients (All patients were CD19+), 28 (48.3%) CALL patients and 26 (44.8%) pre-B patients.
- Patients who did not express the IL-10 showed no pro-B stage. They were 13/24 (54.2%) CALL patients and 11/24 (45.8%) pre-B patients.
- Patients exclusively expressed the IL-10 showed no pro-B stage, 6/11 (54.5%) patients were CALL and 5/11 (45.5%) patients were pre-B.
- Patients expressed the IL-10δ3 variant showed pro-B stage in 4/23 (17.4%) patients, CALL in 9/23 (39.1%) patients and pre-B in 10/23 (43.5%) patients.
- There was no statistically significant relation between Immunophenotyping and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

DNA Index

DNA index was done for 29 samples. While it was <1.16 in 12/12 (100%) patients not expressing the IL-10, 1/2 (50%) patient exclusively expressed the IL-10 and 12/15 (80%) patients expressed the IL-10δ3 variant, the DNA index was ≥1.16 in 1/2 (50%) patients exclusively expressed the IL-10 and 3/15 (20%) expressed the IL-10δ3 variant. No patients who did not express the IL-10 were with DNA index ≥1.16. There was no statistically significant relation between DNA index and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Serum LDH (U/L)

The mean value of serum LDH was 2207.7 u/L in patients not expressing the IL-10, 1779.5 u/L in patients exclusively expressed the IL-10 and 2444.25 u/L in patients who expressed the IL-10δ3 variant. There was no statistically significant relation between Serum LDH and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Response to Therapy

Complete remission was achieved in 21/24 (87.5%) patients not expressing the IL-10, 11/11 (100%) patients exclusively expressed the IL-10 and 23/23 (100%) patients expressing the IL-10δ3 variant. Induction failure occurred in 2/24 (8.3%) patients not expressing the IL-10. Only 1/24 (4.2%) patients not expressing the IL-10 died during induction remission (induction death). There was no statistically significant relation between the response to therapy and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Monoclonal Antibodies used in Immunophenotyping

- CD19 was positive in 19/24 (79.2%) patients not expressing the IL-10, 7/11 (64.6%) patients exclusively expressed the IL-10 and 21/23 (91.3%) patients expressed the IL-10δ3 variant.
- CD10 was positive in 18/24 (75%) patients not expressing the IL-10, 6/11 (54.5%) patients exclusively expressed the IL-10

and 17/23 (73.9%) patients expressed the IL-10δ3 variant.

- CD22 was positive in 18/24 (75%) patients not expressing the IL-10, 7/11 (64.6%) patients exclusively expressed the IL-10, and 18/23 (78.3%) patients expressed the IL-10δ3 variant.
- CD34 was positive in 5/24 (20.8%) patients not expressing the IL-10, 2/11 (18.2%) patients exclusively expressed the IL-10 and 11/23 (47.8%) patients expressed the IL-10δ3 variant.
- MHC CLASS II was positive in 15/24 (62.5%) patients not expressing the IL-10, 6/11 (54.5%) patients exclusively expressed the IL-10 and 16/23 (69.6%) patients expressing the IL-10δ3 variant.

There was no statistically significant relation between CD19, CD10, CD22, CD34 & MHC CLASS II expression and the expression of IL-10 and/or IL-10 isoform, IL-10δ3.

Significance of Immunopheno-typing, CD34 positivity, and Event-Free Survival in Children With B-cell precursor (BCP)-ALL expressing IL-10δ3 compared to non-expressing IL-10δ3 (Table 4).

Table 4: Immunophenotyping, CD34 Positivity, and Event-Free Survival in Pediatric B-Cell Precursor (BCP)-ALL Studied for IL-10δ3 Expression

	IL-10δ3 Expression		Total	P Value.
	None IL-10δ3 (n=35)	With IL-10δ3 (n=23)		
Immunophenotype				
Pro B	—	4(17.4%)	4(6.9%)	0.034
CALL	19(54.3%)	9(39.1%)	28(48.3%)	
Pre B	16(45.7%)	10(34.5%)	26(44.8%)	
CD34 Positivity ±				
No	28(80%)	12(52.2%)	40(69%)	
Yes	7(20%)	11(47.8%)	18(31%)	
EFS (months)				
Median (range)	9 (0-50.5)	41.5 (1-70)		0.001

Significant differences were assessed by $\chi 2$.
±Significant differences were assessed by $\chi 2$ and confirmed by Fisher's Exact Test.
Significant differences were assessed by Mann-Whitney U test.

- Patients who did not expressed the IL-10δ3 showed no pro-B stage. CALL in 19/35 (54.3%) patients and pre-B in 16/35 (45.7%) patients. Patients expressing the IL-10δ3 showed pro-B stage in 4/23 (17.4%) patients, CALL in 9/11 (39.1%) patients and pre-B in 10/23 (34.5%) patients.
- Immunophenotyping between cases expressing IL-10δ3 compared to non-expressing IL-10δ3 was statistically significant (P= 0.034).
- cases expressing IL-10δ3 showed more CD34 positivity (47.8%) compared to non-expressing IL-10δ3 (20%) and that was statistically significant (P= 0.025).
- The median event-free survival (EFS) of patients who did not expressed the IL-10δ3 was lower than in patients expressing the IL-10δ3 (9 and 41.5 months, respectively). EFS was statistically highly significant (P= 0.001).
- Cases expressing IL-10δ3 had a significantly better probabilities of event-free survival (pEFS) (P= 0.014) at five years compared with patients non expressing IL-10δ3

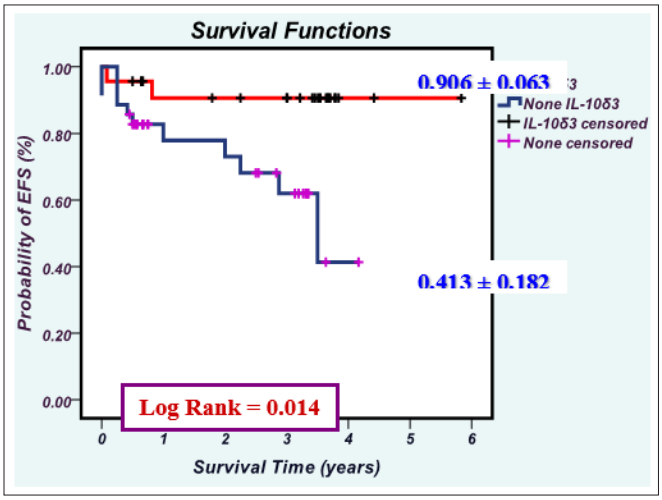

Figure 5: Correlations Between IL-10δ3 Expression and Probabilities of Event-Free Survival (pEFS) in Pediatric B-cell Precursor (BCP) acute Lymphoblastic Leukemia

Discussion

Interleukin-10 (IL-10) is a multifunctional TH-2 cytokine participating in the development and progression of various tumors [7]. The gene encoding IL-10 is located on chromosome 1 (1q31–1q32). It was originally discovered as a powerful inhibitor of inflammatory and immune responses, partly via its inhibition of some macrophage responses. IL-10 can also inhibit tumor-induced angio-genesis, and a wealth of evidence is accumulating that IL-10 carries some immunostimulant properties [9]. There are a number of reports describing elevated levels and expression of IL-10 in patients with particular cancers, including malignant melanoma, prostate cancer, non-Hodgkin's lymphoma, and multiple myeloma. These elevated levels have been reported both in the serum and/or tumor lesions [14-22].

Most cases of acute lymphoblastic leukemia (ALL) arise from malignant transformation of B cell precursors in the bone marrow. The interaction of bone marrow environment, cytokine signaling and the controlled expression of transcription factors guide the differentiation and proliferation of the uncommitted progenitor cell. Interleukin-10 (IL-10) displays a broad spectrum of biologic activities, not only in mature immune cells, including immunosuppressive, anti-inflammatory and B-cell–stimulating properties, but also in the differentiation of immature hematopoietic cells. In addition to these physiological features in normal hematopoiesis, IL-10, like other cytokines or their receptors, has been proposed to promote pathogenesis of leukemic cells. Studies confirmed the secretion of IL-10 by leukemic cells in bone marrow specimens of children with relapsed ALL. Also, pre-treatment serum levels of IL-10, IL-12 and IL-10/IL-12 balance in children with tissue sarcomas (STS), Hodgkin's lymphomas (HL) and acute lymphoblastic leukemia (ALL) might be of value as additional prognostic tools to predict the response to therapy and probability of event free survival (EFS) and overall survival (OS) [23-26].

Many cytokines, include IL-4, IL-6, IL-10 and IL-15, were found to have multiple isoforms derived from different spliced mRNA transcripts [24,27-30]. These cytokine isoforms generally have different affinities to their receptors, contributing to generating different biological signals. In the case of IL-10, a new splicing-derived IL-10 variant (termed IL-10δ3) that lacked the entire exon

3 (IL-10δ3; 153 bp [nucleotides, 226 to378, corresponding to amino acids 72 to 126]) was reported. The in-frame splice variant resulting from exon skipping of exon 3 (GT-AG common splicing consensus sequences) does not cause alteration of the translational reading frame [24]. Both IL-10 and its isoform IL-10δ3 can be detected from acute lymphoblastic leukemia (ALL) samples. It was found that IL-10 was associated with the progression of ALL, while IL-10δ3 was linked to good response to therapy and better clinical outcomes [24]. These data demonstrated different roles of cytokine isoforms in modulating signaling transduction.

The involvement of IL-10 expression in childhood ALL has been suggested in various studies [24,25,31,32]. At disease recurrence, leukemic cells of patients with B-cell precursor (BCP)-ALL showed higher expression of IL-10 mRNA when compared with B-cell precursor (BCP)-ALL at first presentation. Moreover, levels of cytokines/cytokine receptors (C/CR) expression were dependent on the differentiation stage of ALL cells [25]. IL-10 gene polymorphism studies suggested a correlation between the IL-10 genotype and prednisone response in childhood ALL. Patients displaying the IL-10 G/G genotype might have a lower risk for poor prednisone response [12].

Interleukin 10 (IL-10) had often controversially been discussed in the literature. for example, did not find a significant correlation of IL-10 levels with 2-yr survival rate, complete remission (CR) rate, or relapse rate in acute leukemia, whereas found that IL-10 concentration was significantly higher in serum of children with acute lymphoblastic leukemia at the time of diagnosis when compared to controls as well as the patients during remission phase. Also, found that IL-10 was not predictive of clinical outcomes (CR, survival, and EFS) in newly diagnosed acute myeloid leukemia and high-risk myelodysplastic syndromes [33,34].

On the other hand. reported increased circulating IL-10 serum levels in gastric, colon, and renal-cell cancer patients. IL-10 serum levels commonly returned to normal in radically resected patients. Persistently elevated IL-10 serum levels after surgery predicted tumor recurrence. Moreover, a further significant increase in IL-10 serum levels has been observed in non-responders to chemotherapy of the mentioned non hematopoietic malignancies [35-42].

Among 58 patients with BCP-ALL there were 11 (18.9%) relapses, six (25%) of the 24 cases not expressing the IL-10, three (27.3%) of the 11 cases exclusively expressed the IL-10 while only two (8.7%) of 23 cases expressed the IL-10δ3 variant developed relapse. Similar to our findings other reports suggested that the splicing-derived IL-10 isoforms, IL-10δ3 may modulate IL-10–mediated biologic effects and therapeutic efficacy in lymphatic disease. Also, expression of L-10δ3 seemed to be a positive prognostic feature in childhood ALL [24]. Our data showed a possible modulatory role of IL-10δ3 particularly in the decreased incidence of relapse; however the difference was not statistically significant ($P > 0.2$). The incidence of relapse in patients expressing IL-10δ3 was approximately 2 time less when compared to patients only expressing the normal IL-10 transcript and/or patients expressing IL-10 below detection limit (Figure. 2). In our study, none of the 4 patients expressing IL-10δ3 as a sole variant relapsed whereas, reported that no sample of Relapsed Childhood Acute Lymphoblastic Leukemia expressed IL-10δ3 exclusively [24].

Regarding the immunophenotype-typic expression of CD34 in B-cell precursor (BCP)-ALL, in our study we found that CD34 positivity was higher in patients displaying IL-10δ3 expression (47.8%) than in other patients; however the difference had reached the statistical significance only when compared to patients not expressing IL-10δ3 (20%; $P=0.025$) and was border-line when compared to patients expressing only the normal IL-10 transcript and/or patients expressing IL-10 below detection limit (18.2% and 20.8%, respectively; $P=0.08$). Initial studies associated CD34 expression with a worse prognosis and yet it was higher in patients displaying IL-10δ3 expression and with good outcome, then IL-10δ3 may have a counter acting effect. On the other hand, different studies found association between CD34 antigen expression in B-lineage childhood ALL and the presence of favorable presenting features: age between 1 to 10 years, white race, absence of CNS leukemia, low serum lactate dehydrogenase level, leukemic cell CD10 expression, and DNA index ≥ 1.16. Similar conclusions were obtained in the study from the St Jude's Children's Research Hospital [43-47].

Pro-B ALL is one of the most immature ALL subtypes, characterized by the expression of CD19, cytoplasmic (cy) or membrane CD22, CD24 and cyCD79a, and by the absence of CD10, cy and surface immunoglobulin (Ig). A relevant fraction (<30%) of pro-B ALL cases are associated with the t (4;11) (q22; q23) translocation that fuses the ALL1 (MLL/HRX/Hrtx1) gene at 11q23 to the AF4 (FEL) gene at the 4q22. This genetic abnormality is also frequently associated with the expression of myeloid antigens and hybrid phenotypic features in ALL [48]. In childhood acute lymphoblastic leukemia, the pro-B immunophenotype is often associated with unfavorable clinical features such as age less than 1-year, high leukocyte counts, massive hepatosplenomegaly, and central nervous system (CNS) disease at diagnosis. Nevertheless, reported that pro-B-ALL had the highest complete remission (CR) rate among the four subtypes of B-ALL, then cALL [49-53].

In culture, IL-7 and IL-10 are potent growth factors for pro-B cells. IL-10 potently and directly promotes the earliest stage of B-cell development from uncommitted murine bone marrow progenitor cells in combination with flt3 ligand (FL), and IL-7, whereas it inhibits growth of CD19+ pro-B cells implicating a dual role of IL-10 in early B-cell development [54,55].

Although the number of patients in certain subgroups was limited (patients with pro-B), our data suggest an association of pro-B stage in childhood B-cell precursor (BCP)-ALL and IL-10 expression. In our study, the 4 patients with pro-B immunophenotype (all patients were CD19 positive) expressed IL-10δ3. Interestingly, one of the cases was a pro B immunophenotype, harboring t (4;11) rearrangement, a situation that is known to be of poor prognosis, yet the patient had an event free course, EFS and OS of 27 months. Among 58 patients with BCP-ALL there were only 3 non-responders, all of them not expressing the IL-10. Two (8.3%) of 24 cases had Induction failure and one (4.2%) case died during induction (induction death). Response to therapy has been shown to be the most significant predictor of outcome for children with ALL at first presentation. In contrast to [24]. reporting that analysis of clinical relevance revealed a strong correlation between IL-10δ3 expression and response to therapy ($P= 0.001$), our results found none ($P > 0.3$). The difference between our results and other reports may be due to the time bone marrow (BM) samples were collected. Our bone marrow (BM) samples were collected only from children with B-cell precursor (BCP)-ALL at diagnosis, whereas in other studies bone marrow samples were collected from children with first relapse ALL (T-ALL and B-ALL) [55,56].

Contrary to our results, identified interleukin 10 expression as a significant adverse prognostic indicator in childhood BCP-ALL [32]. The event-free survival (EFS) of patients expressing IL-10 mRNA in high quantity was significantly lower compared with patients expressing low IL-10 mRNA. However, our study had stated that the probabilities of event-free survival (pEFS) of patients expressing only the normal IL-10 transcript compared with patients expressing the IL-10 below detection limit was not significant ($P > 0.8$) [57-61].

Also, results indicated that, the prognostic impact of the IL-10 and IL-10 isoform, IL-10δ3, expression was not outweighed through correlation with traditional risk criteria (age, WBC, DNA index) [24]. reporting that at relapse, no statistically significant differences in expression of IL-10δ3 were found regarding the duration of first complete remission. An important finding from this work was a possible trend toward a favorable outcome, with significantly better probabilities of event-free survival (pEFS) at five years in pediatric patients with B-cell precursor (BCP)-ALL was likely to be related to IL-10δ3 expression ($90.6 \pm 6.3\%$; $P=0.047$) compared to patients with exclusive or no expression of IL-10 at first complete remission. On other hand, reported that patients with IL-10δ3 expression in leukemic cells had a significantly better pEFS (0.43 v 0.09; $P=0.01$) at five years compared with patients expressing only the normal IL-10 transcript at first relapse (second complete remission) [24].

In analysis of Interleukin-10 Splicing Variants expression in relapsed childhood acute lymphoblastic leukemia (ALL), reported that IL-10 may play an active role in relapse of ALL by supporting chemoresistance and inhibition of immunocompetent cells [24]. Moreover, IL-10 isoform may have an important function in modulating the biologic activity of normal IL-10. This may account for the less aggressive biologic behavior of IL-10δ3 expressing leukemic cells. It seems that the exclusion of exon 3 by exon skipping leads to altered ligand receptor interactions with decisive functional consequences, including the change of receptor binding affinity, but this remains to be determined. In the opinion of the biologic effects could result from different activities of the splicing-derived isoform with respect to IL-10 receptor binding activity and, in some cases, also from competitive interactions between coexpressed isoforms [24].

Our report showed some disagreement with assumptions that the biologic effects could result from competitive interactions between co expressed isoforms The features of the 4 (BCP)-ALL Patients who expressed IL-10δ3 exclusively, by itself, could explain this conflict for more than one reasons: 1) In our study, the 4 patients expressing IL-10δ3 as a sole variant were associated with better event-free survival. Moreover, none of them relapsed. 2) Our study had found no significant differences in rate of relapse and\ or the probabilities event-free survival (pEFS) between patients expressing only the normal IL-10 transcript compared with patients expressing the IL-10 below detection limit [24].

Hence, this work provided data further supporting the positive prognostic feature of IL-10 isoform, IL-10δ3, expression in a series of childhood B-cell precursor (BCP)-ALL and the probability of preventing and/or decreasing the incidence of relapse. Our data were in agreement with those of Wu and co-workers [24]. We reported the detection of IL-10 isoform, IL-10δ3, expression was proven significant for the event free survival. CD34 positivity – with better prognosis– was higher with IL-10 isoform, IL-10δ3, expression. Finally, our results revealed a possible correlation between pro-B stage and IL-10 isoform, IL-10δ3, expression.

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